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## Nanotechnologies — High throughput screening method for nanoparticles toxicity using 3D model cells

*Nanotechnologies — Méthode de criblage à haut débit de la toxicité des nanoparticules utilisant des systèmes cellulaires 3D*

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# Contents

Page

<b>Foreword</b> .....	<b>iv</b>
<b>Introduction</b> .....	<b>v</b>
<b>1 Scope</b> .....	<b>1</b>
<b>2 Normative references</b> .....	<b>1</b>
<b>3 Terms and definitions</b> .....	<b>1</b>
<b>4 Background</b> .....	<b>2</b>
4.1 General.....	2
4.2 Effects of optical properties of NPs on in vitro cell viability assays.....	2
4.3 New assay platform for in vitro toxicity screening of NPs diminishing optical interference.....	4
4.4 Characteristics of 3D model cells.....	7
4.5 Cell viability in response to NPs assessed using 3D model cells on a pillar insert.....	9
4.6 Cellular uptake of NPs using 3D model cells on a pillar insert.....	13
4.7 Discussion of alternative strategies to evaluate in vitro toxicity testing of NPs.....	16
<b>5 Methods for cell viability screening of NPs using 3D-model cells</b> .....	<b>17</b>
5.1 General.....	17
5.2 Cell culture.....	17
5.3 Preparation of the pillar insert for in vitro screening.....	17
5.4 Encapsulation of cells on a micropillar chip to generate 3D-model cells.....	18
5.5 NPs sample preparation.....	18
5.6 Exposing 3D-model cells to NPs.....	18
5.7 Cell viability analysis using a WST assay.....	19
5.8 Cell viability analysis using live-cell imaging.....	19
<b>Bibliography</b> .....	<b>21</b>

## Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see [www.iso.org/directives](http://www.iso.org/directives)).

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For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT), see [www.iso.org/iso/foreword.html](http://www.iso.org/iso/foreword.html).

This document was prepared by Technical Committee ISO/TC 229, *Nanotechnologies*.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at [www.iso.org/members.html](http://www.iso.org/members.html).

## Introduction

With an increasing number of nano-products including nanoparticles (NPs), potential exposure of consumers to NPs has increased. Therefore, the human and environmental impacts of NPs have recently emerged as an issue. High-throughput screening (HTS) approaches are often used for NPs toxicity screening. However, there are still limitations to provide the reproducible and reliable results based on a HTS method. To assess the potential toxicity of manufactured or engineered NPs, traditional in vitro toxicity studies have been performed using a surface attached two-dimensional (2D) culture system. 2D assays for cellular metabolic activity, cytotoxicity, or oxidative stress have been widely used in the first stage of hazard evaluation. However, several problems were encountered during assay validation, ranging from particle agglomeration in biological media to optical interference with the assay platform. There are ISO documents on the cytotoxic effects of NPs using cell viability assays and detection of reactive oxygen species (ROS) levels, but they can be applicable for a few classes of NPs that are well-dispersed in the media. Additionally, reagents used in the assays can interact with tested NPs or interfere with spectrophotometric reading.

This document describes a new assay platform, consisting of three-dimensional (3D) arrangement of cells on pillar inserts to evaluate cell viability and diminish artefacts arising from optical interferences and NP reactivity with assay components.

This document aims to overcome the optical interference of NPs and obtain reliable and reproducible cell viability results. The 3D-model cells are exposed to fresh cell viability reagent by simply transferring and immersing the pillar insert from one well to another well without optical interference from the NPs. In addition, 3D-model cell culture approaches facilitate cell-cell interactions and enhance cell-to-cell or cell-to-extracellular matrix (ECM) adhesion/signalling, ultimately leading to the expression of phenotypic proteins/genes and the formation of in-vivo tissue-like morphology. It generates uniform cell-containing hydrogel droplets on the pillar insert and allows to easily change cell growth media or expose 3D-model cells to analytical reagents by immersing the tip of the pillar insert in different reaction plates.

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# Nanotechnologies — High throughput screening method for nanoparticles toxicity using 3D model cells

## 1 Scope

This document describes a method for high throughput evaluation of cytotoxic response of 3D model cells exposed to NPs without optical interference.

The method in this document is intended to be used in biological testing laboratories that are competent in the culture and growth of cells and the evaluation of cytotoxicity of NPs using 3D-model cells.

This method applies to materials that consist of nano-objects such as nanoparticles, nanopowders, nanofibres, nanotubes, and nanowires, as well as aggregates and agglomerates of these materials.

## 2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO/TS 80004-2, *Nanotechnologies — Vocabulary — Part 2: Nano-objects*

## 3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO/TS 80004-2 and the following apply.

ISO and IEC maintain terminology databases for use in standardization at the following addresses:

- ISO Online browsing platform: available at <https://www.iso.org/obp>
- IEC Electropedia: available at <https://www.electropedia.org/>

### 3.1

#### **agglomerate**

collection of weakly bound particles or aggregates or mixtures of the two where the resulting external surface area is similar to the sum of the surface areas of the individual components

Note 1 to entry: The forces holding an agglomerate together are weak forces, for example, van der Waals forces, or simple physical entanglement.

Note 2 to entry: Agglomerates are also termed secondary particles, and the original source particles are termed primary particles.

[SOURCE: ISO/TS 80004-2:2015, 3.4, modified — "weakly or medium strongly bound particles" has been replaced with "weakly bound particles or aggregates or mixtures of the two".]

### 3.2

#### **dispersion**

microscopic multi-phase system in which discontinuities of any state (solid, liquid or gas: discontinuous phase) are dispersed in a continuous phase of a different composition or state

Note 1 to entry: If solid particles are dispersed in a liquid, the dispersion is referred to as a suspension. If the dispersion consists of two or more liquid phases, it is termed an emulsion. A super emulsion consists of both solid and liquid phases dispersed in a continuous liquid phase.

[SOURCE: ISO 19007:2018, 3.2]

### 3.3

#### **nano-object**

material with one, two or three external dimensions in the *nanoscale* (3.5)

Note 1 to entry: This is a generic term for all discrete nanoscale objects.

[SOURCE: ISO/TS 80004-2:2015, 2.2, modified — "discrete piece of" has been added to the definition and Note 1 to entry has been replaced.]

### 3.4

#### **nanoparticle**

NP

*nano-object* (3.3) with all three dimensions in the *nanoscale* (3.5)

Note 1 to entry: If the lengths of the longest to the shortest axes of the nano-object differ significantly (typically by more than three times), the terms nanorod or nanoplate are intended to be used instead of the term nanoparticle.

### 3.5

#### **nanoscale**

size range from approximately 1 nm to 100 nm

Note 1 to entry: Properties that are not extrapolations from a larger size will typically, but not exclusively, be exhibited in this size range. For such properties, the size limits are considered approximate.

Note 2 to entry: The lower limit in this definition (approximately 1 nm) is introduced to avoid single and small groups of atoms from being designated as *nano-objects* (3.3) or elements of nanostructures, which can be implied by the absence of a lower limit.

### 3.6

#### **high-throughput screening**

method that comprises the screening of a large number of chemicals via automation, miniaturized assays and large-scale data analysis

Note 1 to entry: This protocol can be applied to screen the toxicity of NPs based on 96-well plate or 532 microchip.

## 4 Background

### 4.1 General

With the increase in the number of consumer products containing NPs, potential exposure to NPs has increased, and potential human and environmental hazards of NPs have emerged. To assess the effects of NPs, a high-throughput screening method to evaluate cell viability following exposure to NPs is needed. High-throughput approaches have been used to screen for toxicity of manufactured NPs<sup>[1]</sup>.

### 4.2 Effects of optical properties of NPs on in vitro cell viability assays

NPs possess linear/nonlinear optical absorbance and photoluminescence emission<sup>[2][3]</sup>. Because of their physicochemical and optical properties, NPs are used in various fields for disease diagnoses or as industrial products. Most NPs exhibit the optical properties in a wide range of absorbance wavelengths, where optical interference can be pronounced in cell viability assays based on absorbance read-outs.

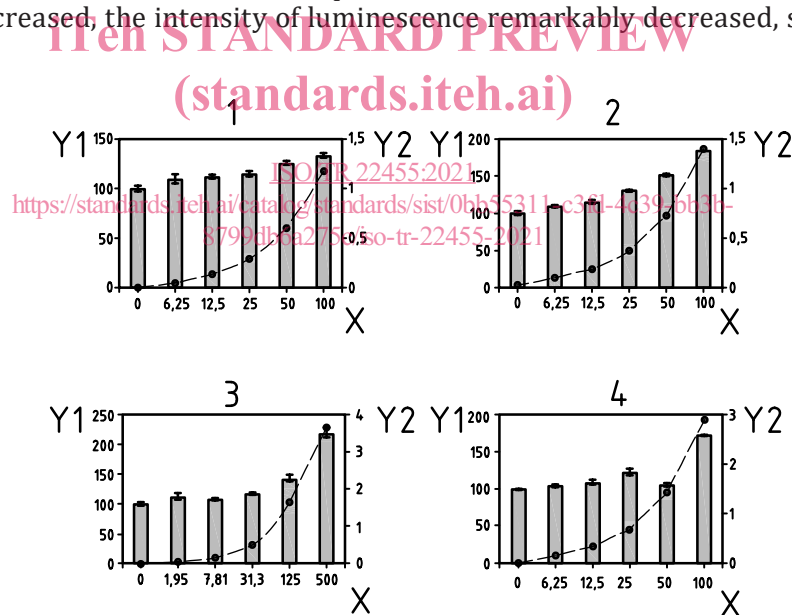
To assess the potential toxicity of manufactured or engineered NPs, traditional in vitro toxicity studies have been performed using 2D model culture systems. During validation of 2D-model assays, several problems, for example, particle agglomeration in biological media and optical interference with the assay system, were encountered<sup>[4][5]</sup>. In traditional cell viability assays, colorimetric detection is generally used, and luminescent and fluorescent detection methods have been also applied to evaluate the cell viability assay. As shown in [Table 1](#), some NPs such as the Ag NPs show an optical absorption at the wavelengths where the colorimetric assays are monitored.



**Table 1 — Range of the wavelengths of conventional cell viability assays**

Assay	Wavelength used for measurement
	nm
WST-1	420 to 480
XTT	450 to 500
WST-8	450 (450 to 490)
MTS	490 (450 to 540)
NRU	540
MTT	570 (500 to 600)

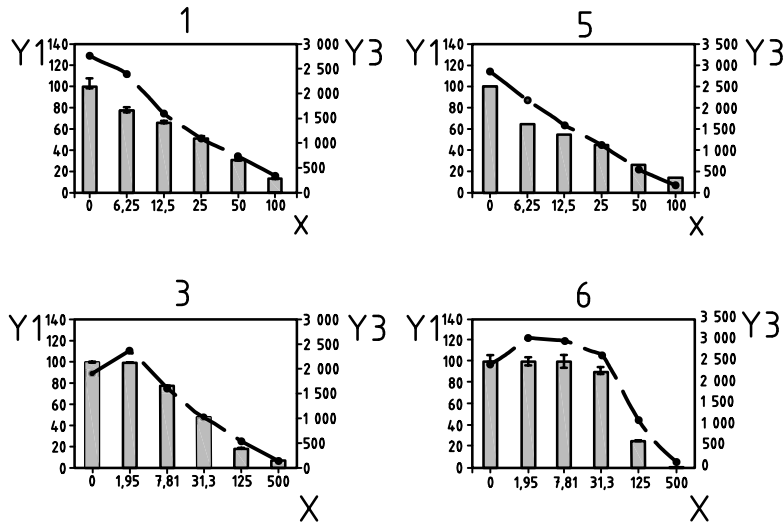
Representative cell viability assays include water-soluble tetrazolium (WST), 2,3-Bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide salt (XTT), 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS), neutral red uptake (NRU), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assays as well as ATP detection based on luminescence, but the optical properties of NPs can influence all of these detection methods. The effects of the optical absorbance of NPs (Ag, silver-shelled gold (Au@Ag), and iron (II, III) oxide (Fe<sub>3</sub>O<sub>4</sub>) NPs and single-wall carbon nanotube (SWCNT) on cell viability were evaluated as shown in [Figure 1, a](#)). The absorbance increased according to the concentration of NPs. Although some remedies, such as multiple washing steps, NPs attached to the cultured cells can still influence the optical absorption reading. These optical properties can lead to false positive or false negative results<sup>[6]</sup>. The luminescence detection method is also vulnerable to the optical interference of NPs, as shown in [Figure 1, b](#)). As the number of NPs increased, the intensity of luminescence remarkably decreased, showing false positive cytotoxicity.



#### a) Colorimetric detection using CCK-8 (Cell Counting Kit-8) reagent (Dojindo)<sup>1)</sup>

CCK-8 (Cell Counting Kit-8) reagent (Dojindo) is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

1) CCK-8 (Cell Counting Kit-8) reagent (Dojindo) is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.



**b) Luminescence detection using CellTiter-Glo® reagent (Promega)<sup>2)</sup>**

CellTiter-Glo® reagent (Promega) is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

**Key**

- X concentration (µg/ml)
- Y1 cell viability (%)
- Y2 absorbance
- Y3 luminescence
- █ cell viability
- background absorption
- background values

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1 Ag  
2 Ag-shelled Au  
3 Fe<sub>3</sub>O<sub>4</sub>  
4 SWCNT  
5 Au

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**Figure 1 — Effects of optical properties of NPs on cell viability**

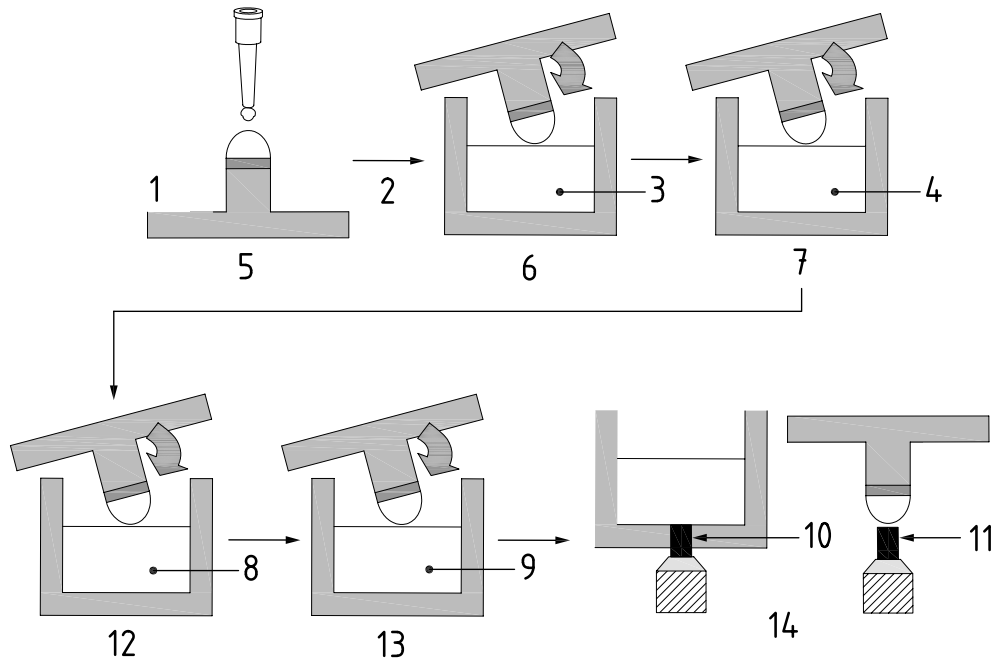
A549 cells were treated with NPs under the pre-dose finding ranges for 24 h, and cell viability was measured using the colorimetric and luminescence detection method. The absorbance of NPs without cells was measured as the background. The left axis represents the relative cell viability, and the right axis represents the absorption values at 450 nm (A) and luminescence (B). For colorimetric detection using CCK-8 (Cell Counting Kit-8) reagent (Dojindo) in panel (A), the cell viability and background absorption are represented with bars and dashed lines respectively. For luminescence detection using CellTiter-Glo® reagent (Promega) in panel (B), the cell viability and background values are represented with bars and dashed lines respectively. SWCNT, single-walled carbon nanotube; QD, quantum dot.

**4.3 New assay platform for in vitro toxicity screening of NPs diminishing optical interference**

3D-model cells on a pillar insert are used to evaluate the cell viability while minimizing artefacts such as those associated with optical absorption and undesirable reactions with an assay reagent. In the use of these platforms, the 3D-model cells are exposed to fresh cell viability reagent by simply transferring and immersing the pillar insert from a column of a well to another column of that well without the optical interference of the NPs, and the schematic flow is shown in Figure 2. This platform allows to easily exchange cell growth media and to expose the 3D-model cells to analytical reagents by immersing

2) CellTiter-Glo® reagent (Promega) is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

the tip of the pillar insert in different reaction plates<sup>[7][10]</sup>. Thus, this method allows high-throughput screening of NPs cytotoxicity by reducing optical interference and reactivity with assay reagents.



**Key**

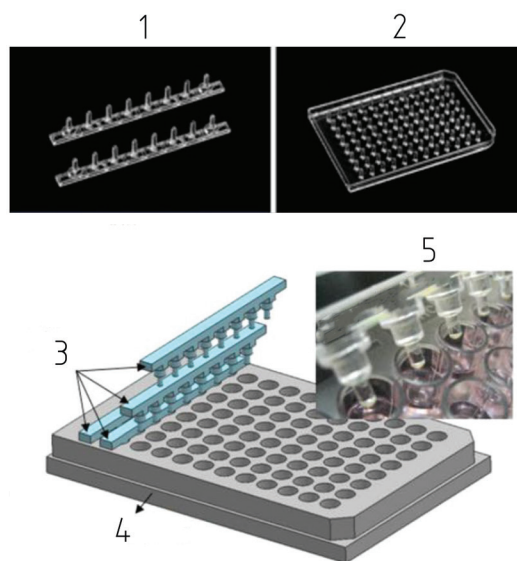
- |                 |   |
|-----------------|---|
| 1 pillar insert | 8 PBS                                       |
| 2 96-well       | 9 reagent                                   |
| 3 growth medium | 10 absorbance                               |
| 4 nanoparticles | 11 fluorescent intensity                    |
| 5 cell seeding  | 12 washing                                  |
| 6 cell culture  | 13 assay                                    |
| 7 treatment     | 14 measurement of cell viability (10 or 11) |

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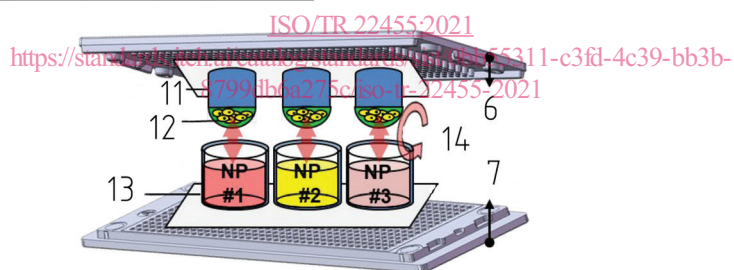
**Figure 2 — Schematic flow of the pillar insert system for evaluating the cell viability of NPs**

The cell-alginate mixture was dispensed onto a pillar insert. The encapsulated 3D cells on the pillar insert were cultured in the medium and then exposed into NPs. The following exposed 3D cells on pillar insert is easily transferred into an independent well with WST-8 or calcein AM reagent and the cell viability can be measured by absorbance or fluorescence.

The method is applicable to 96-well plates and can be extended to 532-well plates microchips (see [Figure 3](#)).



a) 96-well system



b) 532-well microchip system

**Key**

- |   |                  |    |                             |
|---|------------------|----|-----------------------------|
| 1 | 8-pillar strip   | 8  | slide glass (75 mm × 25 mm) |
| 2 | 96-pillar plate  | 9  | top micropillar             |
| 3 | pillar insert    | 10 | bottom microwell            |
| 4 | 96-well plate    | 11 | pillar                      |
| 5 | pillar insert    | 12 | cells in alginate           |
| 6 | micropillar chip | 13 | well                        |
| 7 | microwell chip   | 14 | stamping                    |

NOTE Each pillar insert was immersed into 96 wells or 532 microwells.

**Figure 3 — Schematics of the assay platform to screen the cell viability of NPs based on a 96-well or 532-well microchip system**